



Adenoviral vectors elicit humoral immunity against variable loop 2 of clade C HIV-1 gp120 via “Antigen Capsid-Incorporation” strategy

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ABSTRACT

Adenoviral (Ad) vectors in combination with the “Antigen Capsid-Incorporation” strategy have been applied in developing HIV-1 vaccines, due to the vectors’ abilities in incorporating and inducing immunity of capsid-incorporated antigens. Variable loop 2 (V2)-specific antibodies were suggested in the RV144 trial to correlate with reduced HIV-1 acquisition, which highlights the importance of developing novel HIV-1 vaccines by targeting the V2 loop. Therefore, the V2 loop of HIV-1 has been incorporated into the Ad capsid protein. We generated adenovirus serotype 5 (Ad5) vectors displaying variable loop 2 (V2) of HIV-1 gp120, with the “Antigen Capsid-Incorporation” strategy. To assess the incorporation capabilities on hexon hypervariable region1 (HVR1) and protein IX (pIX), 20aa or full length (43aa) of V2 and V1V2 (67aa) were incorporated, respectively. Immunizations with the recombinant vectors significantly generated antibodies against both linear and discontinuous V2 epitopes. The immunizations generated durable humoral immunity against V2. This study will lead to more stringent development of various serotypes of adenovirus-vectored V2 vaccine candidates, based on breakthroughs regarding the immunogenicity of V2.

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Introduction

Since the beginning of the human immunodeficiency virus type 1 (HIV-1) epidemic, nearly 70 million infections have occurred, and approximately 35 million people have died of AIDS (<http://www.who.int/gho/hiv/en/>). Tremendous progress has been made to combat the HIV-1 epidemic, such as development of new drugs. However, drugs alone have failed to successfully eradicate HIV-1 infections due to the high genetic variation of HIV-1, multidrug resistance, limited access to treatment due to cost of drugs and/or potential side-effects of drugs. Developing a safe, inexpensive, and highly effective HIV-1 vaccine is a priority and remains the unrelenting focus for protection against HIV-1 infection. Among the five HIV-1 vaccine clinical efficacy trials conducted, RV144 demonstrated the most promise with moderate efficacy at 31.2% (Rerks-Ngarm et al., 2009). Primary case-control analyses in RV144 identified two major variables that were associated with risk of HIV-1 infection: the binding of conformational plasma

IgG antibodies to a V1V2 loop of HIV-1 gp120 presented on scaffold protein gp70 (gp70-V1V2) was inversely correlated with risk of HIV-1 infection, while the binding of plasma IgA antibodies to the Env glycoprotein of HIV-1 was directly correlated with significantly higher level of HIV-1 infection (Haynes et al., 2012). Further correlation analyses indicated that plasma IgG antibodies to linear epitopes in the V2 loop also correlated with reduced risk of HIV-1 infection (Gottardo et al., 2013). These statistical findings demonstrated the potential importance of V2 in the immune-mediated protection against HIV-1 infection, and called for further efforts to focus on V2 vaccines.

The V2 loop is located on the tip of the trimeric Env protein (Julien et al., 2013; Pancera et al., 2014; White et al., 2010), and forms a triple spike with V1 loop and V3 loop (Liu et al., 2011). This spike undergoes conformational changes to facilitate HIV-1 entry. Upon the initial binding of gp120 trimer to CD4 presented on susceptible cells, the V1V2 loops spatially rearrange to unmask V3 loop for the subsequent steps necessary for HIV-1 entry (Wilens et al., 2012). The V2 sequence is highly variable across different HIV-1 isolates. This variability contributes to viral evasion from the host immune response. However, the immune-dominant V2 loop has been characterized with several conserved structural elements that contain highly antigenic epitopes. These epitopes are shared

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or specifically targeted by either linear or conformational antibodies against V2, including broadly neutralizing antibodies and others (Gorny et al., 2012; Karasavvas et al., 2012; Mayr et al., 2013; Nakamura et al., 2012). The above bio-characteristics of V2 have suggested that it is necessary and feasible to develop anti-HIV-1 strategies that are cross-clade and can block the viral life cycle at the cellular entry step.

Multiple viral vectors have been evaluated pre-clinically or clinically to deliver HIV-1 immunogens to host immune systems, most of which have demonstrated established antigenicity and immunogenicity at various levels (Baden et al., 2013; Gomez-Roman et al., 2006; Gomez et al., 2012; Gray et al., 2011; Hammer et al., 2013; Rerks-Ngarm et al., 2009). Among these trials, human Ad vectors with different serotypes have been employed, but limited to the expression of HIV-1 genes that are incorporated into early regions of the vectors. In this proof-of-principle study, by utilizing the “Antigen Capsid-Incorporation” strategy (Gu et al., 2013; Matthews et al., 2010) which features directly displaying protein-of-interests on adenoviral capsid proteins, we sought to investigate the incorporation, antigenicity and immunity of V1/V2 proteins on Ad5.

Results

Incorporation of variable loop antigens onto Ad5 vector

In order to demonstrate the feasibility of incorporating various HIV-1 loops onto the Ad5 capsid, the “Antigen Capsid-Incorporation” strategy was employed and two capsid proteins (hexon HVR1 and pIX) were assessed as insertion locales. One truncated V2 sequence and the full length V2 (43aa in red) and V1V2 (24 aa in black and 43aa in red) sequences (Fig. 1A) were designed to generate three recombinant Ad5 vectors, named Ad-HVR1-20aa-V2, Ad-HVR1-V2 and Ad-pIX-V1V2, respectively (Fig. 1B). The three sequences incorporated into the Ad vectors were derived from clade C HIV-1 strain 205 F (Lynch et al., 2011). Physical titers (VP/ml) and infectious titers (IP/ml) were determined, leading to the calculation of VP/IP ratios. A normal VP/IP ratio of unmodified Ad ranges from ~10–30 (Gu et al., 2013), and an acceptable VP/IP ratio for modified vectors is below or around 1000. We observed increased but normal VP/IP ratios for Ad-HVR1-20aa-V2 and Ad-HVR1-V2 at 120.0 and 600.0, respectively, when compared to the Ad vector (ratio of 31.0) (Table 1). These ratios indicated that the two modifications on HVR1 had minimal effects on the fitness of the Ad vectors, and also suggested that longer foreign sequence insertions into the HVR1 would further decrease stability. We observed the VP/IP ratio of Ad-pIX-V1V2 at 326.3 (Table 1), indicating achievement of a stable vector after the long sequence incorporation into the pIX locale.

Variable loop antigens are exposed on Ad5 surface and are antigenic

Native western-blot or whole virus enzyme-linked immunosorbent assay (ELISA) can detect antigens without denaturing their spatial structure. By virtue of this property, we sought to study the exposure and antigenicity of the variable loop antigens on the rescued recombinant vectors. We determined whether exposure of the V1/V2 insertions on the vector capsid surface was similar to the native conformation of the corresponding antigens. A conformational human monoclonal antibody (hMAb) 6.4 C derived from subject Z205F (Lynch et al., 2011) was applied for both immunological methods. Native western blot analyses illustrated that all three capsid-modified vectors Ad-pIX-V1V2, Ad-HVR1-V2 and Ad-HVR1-20aa-V2 retained specific binding to 6.4 C, when compared to the non-incorporated vector Ad (Fig. 2A). In the

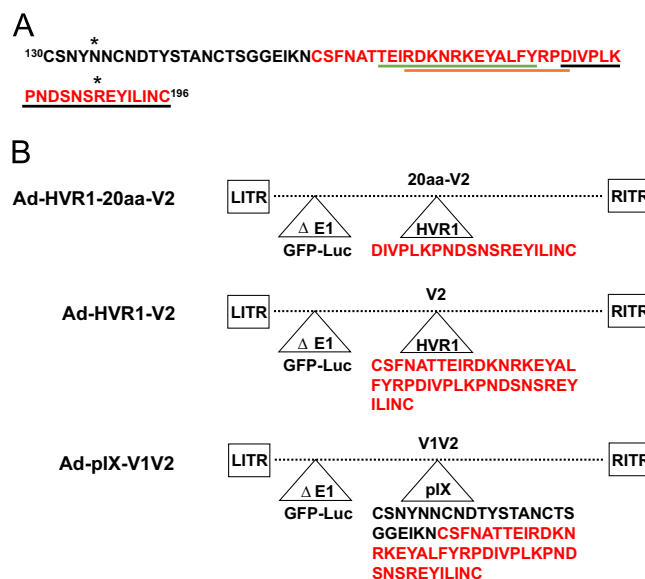


Fig. 1. Generation of Ad5 vectors displaying various lengths of V1/V2 antigens. (A) Full length (67aa) of V1V2 loop of HIV-1 clade C strain Z205F. 24aa in black indicates the V1 loop and 43aa in red indicates the V2 loop. The black underlined sequence indicates the 20aa-V2 epitope incorporated onto adenovirus. The green or orange underlining indicates the most or sub immunodominant domain of Z205F V2 loop according to previous study (Zolla-Pazner et al., 2013). Asterisks at aa¹³⁴ of V1 loop and at aa¹⁸⁹ of V2 loop indicate the key residues required for the generation of the conformational antibody 6.4 C. (B) Utilizing the “Antigen Capsid-Incorporation” strategy allowed successful incorporation of 20aa-V2, full length V2 or full length V1V2 onto hexon HVR1 or pIX locales to generate Ad-HVR1-20aa-V2, Ad-HVR1-V2 or Ad-pIX-V1V2, separately. All incorporated sequences are from HIV-1 clade C Env Z205FPL27MAR03ENV6.3, Genbank accession number GQ485419.

Table 1
Virological properties of Ad5 vectors.

Modified vectors	Strain; Clade	Viral particles (VP/ml)	Infectious particles (IP/ml)	VP/IP
Ad	NA	3.1x10 ¹²	1.0x10 ¹¹	31.0
Ad-HVR1-20aa-V2	Z205F; C	1.2x10 ¹²	1.0x10 ¹⁰	120.0
Ad-HVR1-V2	Z205F; C	2.7x10 ¹¹	4.5x10 ⁸	600.0
Ad-pIX-V1V2	Z205F; C	6.2x10 ¹¹	1.9x10 ⁹	326.3

20aa-V2, full length of V2 and full length of V1V2 were incorporated in hexon HVR1 or pIX of Ad5, to generate recombinant viral vectors Ad-HVR1-20aa-V2, Ad-HVR1-V2 or Ad-pIX-V1V2, respectively.

All V2 sequences of various lengths were selected from HIV-1 clade C Z205FPL27MAR03ENV6.3.

The viral vector fitness was determined by viral particles, infectious particles and corresponding VP/IP ratio.

whole virus ELISA analyses, when compared to Ad, both Ad-HVR1-V2 and Ad-HVR1-20aa-V2 bound significantly to 6.4 C, as $p < 0.01$ (Fig. 2B). Ad-pIX-V1V2 also bound significantly to the antibody, when compared to Ad, as $p < 0.05$ (Fig. 2B). Taken together, the immunologic assays demonstrated that Ad-HVR1-20aa-V2, Ad-HVR1-V2 and Ad-pIX-V1V2 correctly present the variable loop antigens on vector capsid surface.

Vector immunizations induce specific antibodies against linear V2 epitopes

The “prime-boost” regimen was employed by immunizing mice with Ad, Ad-HVR1-20aa-V2, Ad-HVR1-V2 and Ad-pIX-V1V2. Ad was used as a negative control in order to highlight the potential immune responses triggered by V2. The two-week intervals between injections or between injection and bleeding (Fig. 3A)

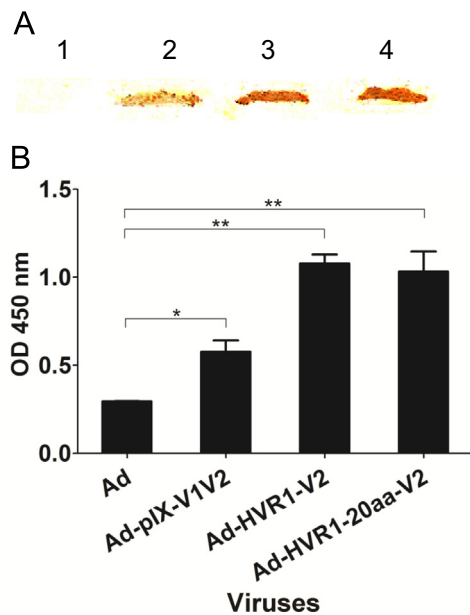


Fig. 2. Evaluation of exposure and antigenicity of V2 antigens on recombinant Ad5 vectors. pIX has approximately one third of the molecular copies of hexon. In order to analyze the V2 antigenicity with equal amount of V2 copies, three times higher dosage of Ad-pIX-V1V2 was needed. (A) Native western-blot analysis was conducted. Ad (lane 1.7×10^9 VP), Ad-pIX-V1V2 (lane 2.21×10^{10} VP), Ad-HVR1-V2 (lane 3.7×10^9 VP) and Ad-HVR1-20 aa -V2 (lane 4.7×10^9 VP) were resolved on SDS-PAGE and transferred onto PVDF membrane. The membrane was blotted with HIV-1 205 F strain-derived hMAb 6.4 C. (B) Whole virus ELISA was employed for the evaluation. Ad (6×10^9 VP/well), Ad-pIX-V1V2 (6×10^9 VP/well), Ad-HVR1-20aa-V2 (2×10^9 VP/well) and Ad-HVR1-V2 (2×10^9 VP/well) were immobilized in ELISA plates. hMAb 6.4 C was applied at 1:1000 to the coated vectors, followed by incubation with goat anti-human IgG-HRP secondary antibody at 1:2000. The values were expressed as the mean \pm standard deviation, representing three independent replicates.

were sufficient to trigger B cell maturation and activation of humoral immune responses (Gu et al., 2013; Matthews et al., 2010). C57BL/6 mice sera from Ad-HVR1-20aa-V2 immunizations were immunologically assessed in sera-based ELISA with coating of the linear 20aa-V2 peptide DIVPLKPNDSNSREYLINC. The data showed increasing binding of 20aa-V2 specific sera to the peptide, when compared to Ad sera, and the binding differences reached significance in both boost and reboost sera, as $p < 0.01$ and $p < 0.05$, respectively (Fig. 3B). Isotype ELISA showed that either IgG2b (boost) or IgG2a (reboost) were identified as dominant in the 20aa-V2 specific sera, compared to the sera from Ad immunizations, as $p < 0.05$ (Fig. 3C). We tried but failed to detect the 20aa-V2 specific immunity in the mice model of Balb/c elicited by Ad-HVR1-20aa-V2 (Data not shown). Balb/c mice sera from Ad-HVR1-V2 immunizations were assessed in sera-based ELISA with coating of the linear V2 peptide EYALFYRPDIVPLKPNDC. Results showed that sera from the immunization group Ad-HVR1-V2, at all three time points (prime, boost and reboost) bound significantly to the linear V2 peptide, when compared to the sera from Ad immunization group, as $p < 0.01$ (prime) and $p < 0.001$ (boost and reboost) (Fig. 4A). The total IgGs specific for linear V2 epitopes were quantitated between 60 μ g/ml and 110 μ g/ml, as post immunization time increased. Isotype ELISA with the linear V2 peptide illustrated that all IgG isotypes (IgG1, IgG2a, IgG2b and IgG3) against linear V2 epitopes were significantly triggered at the boost time point by the administration of Ad-HVR1-V2, as $p < 0.05$ (IgG1 and IgG2b) and $p < 0.001$ (IgG2a and IgG3). The V2-specific IgG1 and IgG2b isotypes were further elicited at the reboost time point, as $p < 0.001$ (Fig. 4B). Ad-pIX-V1V2 was also investigated for its ability to trigger V2-specific humoral immunity using ELISA plates coated with the same linear V2 peptide. The data indicated

that there was not a significant elicitation of total IgGs against linear V2 epitopes (about 20 μ g/ml) at the prime time point, as $p > 0.05$. However, V2-specific total IgGs increased to about 80 μ g/ml at reboost, and was significant at both boost and reboost, as $p < 0.05$ (boost) and $p < 0.01$ (reboost) (Fig. 4C). In Fig. 4D, isotype ELISA with the identical coating peptide showed that all IgG isotypes against linear V2 epitopes except IgG2b were significantly triggered at boost, as $p < 0.05$ (IgG1, IgG2a and IgG3). The significance was further enhanced for IgG2a at reboost, as $p < 0.001$. Moreover, the binding of V2-specific IgG2b to the coated peptide also achieved significance, as $p < 0.05$.

Vector immunizations induce specific antibodies against discontinuous V2 epitopes

Since humoral immune responses to the linear epitopes in V2 were successfully demonstrated in the three recombinant Ad vectors carrying different lengths of V1/V2 sequences on different capsid protein locales, we sought to investigate whether these vectors could also generate humoral immune responses against the discontinuous epitopes in V2. There have been a few V2-specific conformational antibodies that have been isolated from HIV-1 infected subjects and well characterized (Mayr et al., 2013). In this study, a cyclic V2 peptide CEYALFYRPDIVPLKPNDC was synthesized and used for the assessment of both Ad-HVR1-V2 and Ad-pIX-V1V2. Conformation restricted cyclic peptides are normally designed to mimic the native secondary structure of antigens, thus detecting conformational antibodies (Perez et al., 2006). Similar to the total IgGs generated against linear V2 epitopes in Fig. 4A, the conformational total IgGs against V2 were significantly triggered as early as the prime time point ($p < 0.01$) and were enhanced at both boost ($p < 0.001$) and reboost time points ($p < 0.001$) (Fig. 5A). The V2-specific conformational IgG isotypes were also similarly elicited, with IgG1, IgG2a, IgG2b and IgG3 remaining significant regarding the binding to the cyclic peptide (Fig. 5B). The assessment of Ad-pIX-V1V2 was also similar to Fig. 4C, in that conformational total IgGs against V2 were significantly elicited at both boost and reboost, but not the prime time point (Fig. 5C). The V2-specific conformational IgG isotypes were significantly generated, including IgG1, IgG2a, IgG2b and IgG3 (Fig. 5D).

Vector immunizations induce durable V2-specific humoral immunity, but not V2-specific IgG3

To investigate if the V2 vectors could generate durable immune responses specific to the V2 loop, Ad-pIX-V1V2 was selected for this evaluation. Antibodies against both linear and discontinuous V2 epitopes were measured at different time points post reboost by sera-based total IgG ELISAs. In an ELISA with the linear V2 peptide EYALFYRPDIVPLKPNDC, antibodies specific to the linear V2 epitopes from all five time points post reboost (month: 0.5, 1, 2, 3 and 4) were around 60 μ g/ml, with a very slight decrease over time. The anti-V2 antibodies from all time points bound significantly to the linear peptide, as compared to Ad (Fig. 6A). Very similar results were obtained in ELISA with the cyclic V2 peptide CEYALFYRPDIVPLKPNDC (Fig. 6B). Overall, the data suggests that Ad-pIX-V1V2 induced durable humoral immunity against V2. Recent research reported that the IgG3 specific to V2 correlates with lower HIV-1 infection risk, but that IgG3 declines with a half-life at 8.4 weeks (Yates et al., 2014). In this regard, we investigated the half-life of V2-specific IgG3 generated against Ad-pIX-V1V2. Sera-based IgG isotype ELISA with either linear V2 peptide (Fig. 6C) or cyclic V2 peptide (Fig. 6D), both showed that there is significant detection of V2 IgG3 level at 0.5 and 1 month post reboost time points, but this diminished at 2 months post reboost, indicating a shorter half-life in the mice model.

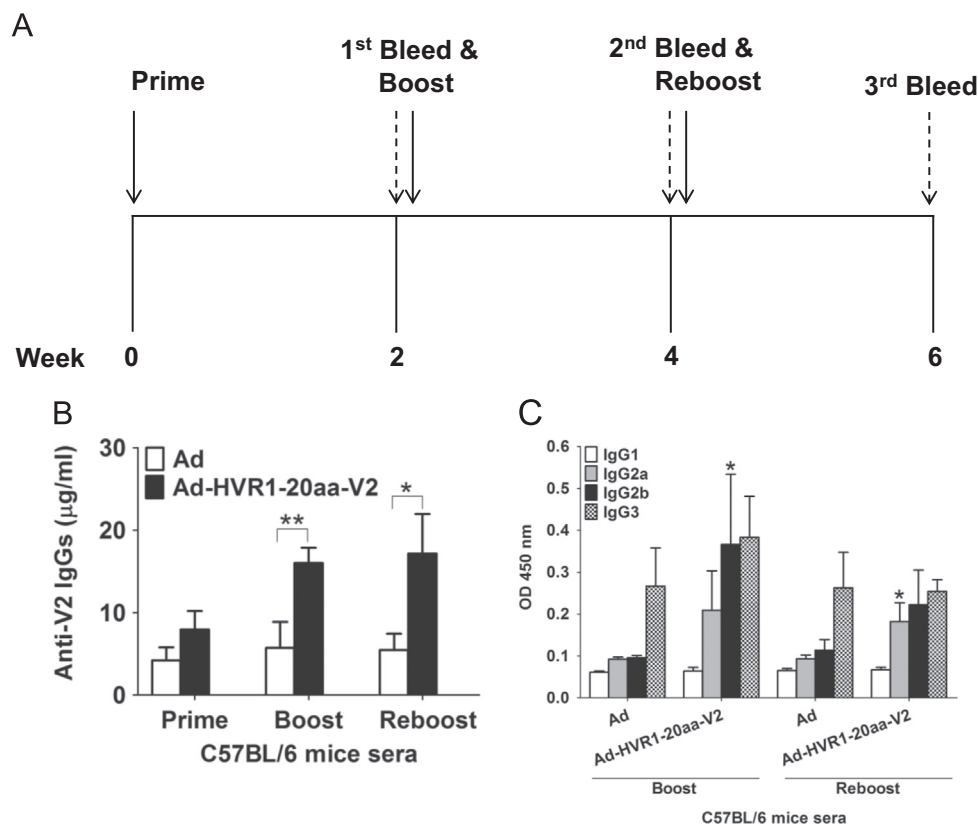


Fig. 3. Schematic of homologous immunization regimen and V2-specific humoral immunity elicited by recombinant Ad vectors. (A) Balb/c mice or C57BL/6 mice were injected with Ad-HVR1-20aa-V2 (Fig. 3), Ad-HVR1-V2 (Figs. 4, 5) or Ad-pIX-V1V2 (Figs. 4, 5), respectively. Ad was used as negative control to highlight the V2-specific immunogenicity of each recombinant vector. The solid lines with arrow denote the time of prime, boost and reboost, the dotted lines with arrow denote the time of bleeding post immunization. (B) C57BL/6 mice sera were collected from immunizations with Ad-HVR1-20aa-V2. Sera were evaluated for anti-V2 IgGs at post prime, boost, and reboost time points. In the sera-based total IgG ELISA, the linear 20aa-V2 peptide was coated to titrate the concentrations of V2-specific total IgGs. (C) V2-specific IgG isotype analysis was performed via ELISA. The 20aa-V2 peptide was coated on ELISA plates, followed by sera from boost and reboost time points. Goat anti-mouse isotypes, IgG1, IgG2a, IgG2b and IgG3 were incubated, followed by incubation with HRP-conjugated donkey anti-goat antibody. All data were expressed as the mean \pm standard deviation, from four independent replicates.

V2-specific mice sera bind to HIV-1 envelope protein

Since the mice sera immunized with the V2 vectors recognize the V2 peptides, it is meaningful to investigate whether the sera also recognize native HIV-1 Env protein. Immunological fluorescence assay (IFA) was utilized for this investigation. In this assay, the positive control hmAb 6.4 C bound specifically and robustly to the HIV-1 Env protein (Z205FPL27MAR03ENV6.3), as indicated by the intensity of the green fluorescence (Fig. 7A). Reboost sera from mice immunized with Ad-HVR1-20aa-V2 showed a similar level of basal binding background to the reboost sera from Ad, indicating the inability of Ad-HVR1-20aa-V2 to elicit antibodies that can recognize the Z205F Env protein (Fig. 7B). However, sera immunized with Ad-HVR1-V2 showed greater binding, as substantial green fluorescence was present at the boost time point, and was further enhanced at the reboost time point (Fig. 7C). This implied that Ad-HVR1-V2 immunization elicited antibodies that can recognize the Z205F Env protein at boost, and generated more antibodies at reboost that fundamentally increased the specific recognition. Ad-pIX-V1V2 also generated antibodies that specifically recognize the Z205F Env protein, as demonstrated by green fluorescence brightness at reboost (Fig. 7D).

Discussion

Over three decades, there has been a continuous effort to develop cell-mediated or antibody-producing HIV-1 vaccines. However, all

clinical trials thus far have failed in achieving efficacy of protection except the RV144 trial (Rerks-Ngarm et al., 2009), although most preclinical vaccine trials have demonstrated antigenicity, immunogenicity and even various protections in animal models (Barouch et al., 2012; Gomez-Roman et al., 2006; Patterson et al., 2004; Ratto-Kim et al., 2012). RV144 and other clinical trials gave the vaccine field a glimmer of promise but also faced problems, such as the lack of potent neutralizing antibodies against HIV-1. Although breakthroughs like the development of artificial Env constructs that mimic the native Env trimeric structure have been achieved, developing additional vaccination strategies against HIV-1 infection is warranted. The V1/V2 domain of HIV-1 gp120 is one hotspot that has attracted increasing attention to develop novel vaccine strategies, because the RV144 trial statistically illustrated the inverse correlation of V1/V2 binding antibodies with HIV-1 acquisition (Haynes et al., 2012). V1/V2 is also attractive as a vaccine antigen because it is involved in mediating HIV-1 entry into host cells (Wilen et al., 2012). The above illustrations support the concept of generating V1/V2-based HIV-1 vaccines. This study as a proof-of-concept illustrated the technical achievement of generating Ad5 vectored V1/V2 vaccine candidates.

Ad vectors have accounted for 22.8% of gene-therapy clinical trials by the end of 2014 (<http://www.wiley.com/legacy/wileychi/genmed/clinical/>). Among the Ad vectors, Ad5 as a vaccine vector has been intensively applied in many preclinical and clinical trials, with proven antigenicity and immunogenicity for different pathogens, including HIV-1 (Gomez-Roman et al., 2006; Gray et al., 2011; Peng et al., 2005), and even protective efficacy (Pandey et al., 2012). The utility of Ad5 is the advantage that the vector can

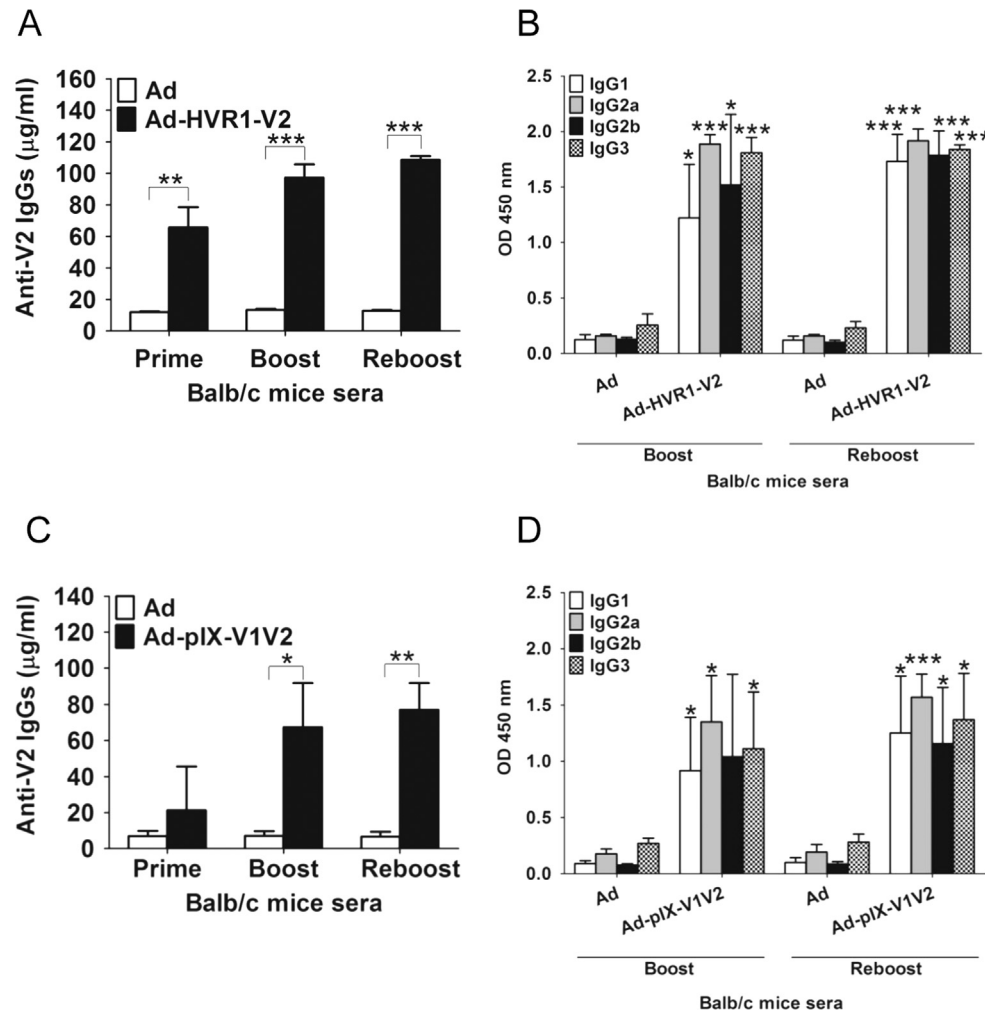


Fig. 4. V2-specific non-conformational IgGs elicited by Ad-HVR1-V2 or Ad-pIX-V1V2. (A) Balb/c mice sera were collected from immunizations with Ad-HVR1-V2. In the sera-based total IgG ELISA, linear V2 peptide was coated to titrate the V2-specific total IgGs (non-conformational) in sera collected from post prime, boost and reboost time points. (B) Corresponding to the total IgG ELISA, linear V2 peptide was coated in the sera-based IgG isotype ELISA. Goat anti-mouse isotypes, IgG1, IgG2a, IgG2b and IgG3 were then applied, followed by incubation with HRP-conjugated donkey anti-goat antibody. (C) Similarly, Balb/c mice sera were collected from immunizations with Ad-pIX-V1V2. Linear V2 peptide was coated in the sera-based total IgG ELISA to titrate the V2-specific total IgGs (non-conformational) in sera collected from post prime, boost and reboost time points. (D) Linear V2 peptide was coated in the sera-based IgG isotype ELISA. Goat anti-mouse isotypes, IgG1, IgG2a, IgG2b and IgG3 were then applied, followed by incubation with HRP-conjugated donkey anti-goat antibody. All data were expressed as the mean \pm standard deviation, from four independent replicates.

be easily engineered, by not only inserting short to long DNA sequences for traditional transgene purposes (Gomez-Roman et al., 2006; Lubeck et al., 1997), but also by incorporating foreign antigens onto the capsid proteins (hexon, pIX, fiber) of the vector via the “Antigen Capsid-Incorporation” strategy. However, a major problem associated with Ad5 as a vaccine vector is the wide existence of pre-immunity to Ad5 in 50–90% of normal adults, which can potentially hamper the traditional transgene efficacy of Ad5 (Zaiss et al., 2009). Strategies have been developed to overcome Ad5 pre-immunity, such as increasing Ad5 vectored vaccine immunization dosages or changing immunization routes (Pandey et al., 2012), using less prevalence serotypes for vaccine vectors (Abbink et al., 2007), and generating Ad5 vectored vaccines via the “Antigen Capsid-Incorporation” strategy (Shiratsuchi et al., 2010). Pre-existing Ad5 seropositivity has been associated with HIV-1 acquisition in the STEP trial (Buchbinder et al., 2008), but not other non-Ad HIV-1 vaccine efficacy trials (Stephenson et al., 2012). Nevertheless in this study, the major rationale was to use Ad5 to generate V1/V2 vaccine candidates via the “Antigen Capsid-Incorporation” strategy and confirm the V2-specific antigenicity/immunogenicity on Ad5.

We previously generated various Ad5 multivalent HIV-1 vaccine vectors (Gu et al., 2013) and attempted to expand on this strategy in the context of antigen capsid-incorporation in combination with V1/V2 presentation. With this strategy, we chose the hexon HVR1 and pIX locales for incorporating V1/V2 sequences. Our results demonstrate that we successfully incorporated different lengths of V2 sequences onto HVR1 and their corresponding VP/IP ratios indicated that these recombinant vectors Ad-HVR1-20aa-V2, Ad-HVR1-V2 were stable (Fig. 1B, Table 1). We also succeeded in incorporating full length V1V2 (67aa) onto pIX to generate Ad-pIX-V1V2, indicating the high plasticity on pIX (Fig. 1B). The corresponding VP/IP ratio also demonstrated stability of the chimeric vector (Table 1). This incorporation strategy also demonstrated exposure and antigenicity of V2 on Ad5 (Figs. 2A, 2B), suggesting that these chimeric Ad5 vectors can also be used to evaluate V2 immunogenicity.

The primary reason for evaluating both mouse strains (C57BL/6 and Balb/c) initially in this study is to demonstrate that V2-specific responses can be generated in both mouse models, which provides a level of variability that might be similar to immune responses seen in two individuals. Homologous repeat immunizations with each vector, Ad-HVR1-20aa-V2, Ad-HVR1-V2 or Ad-pIX-V1V2, demonstrated

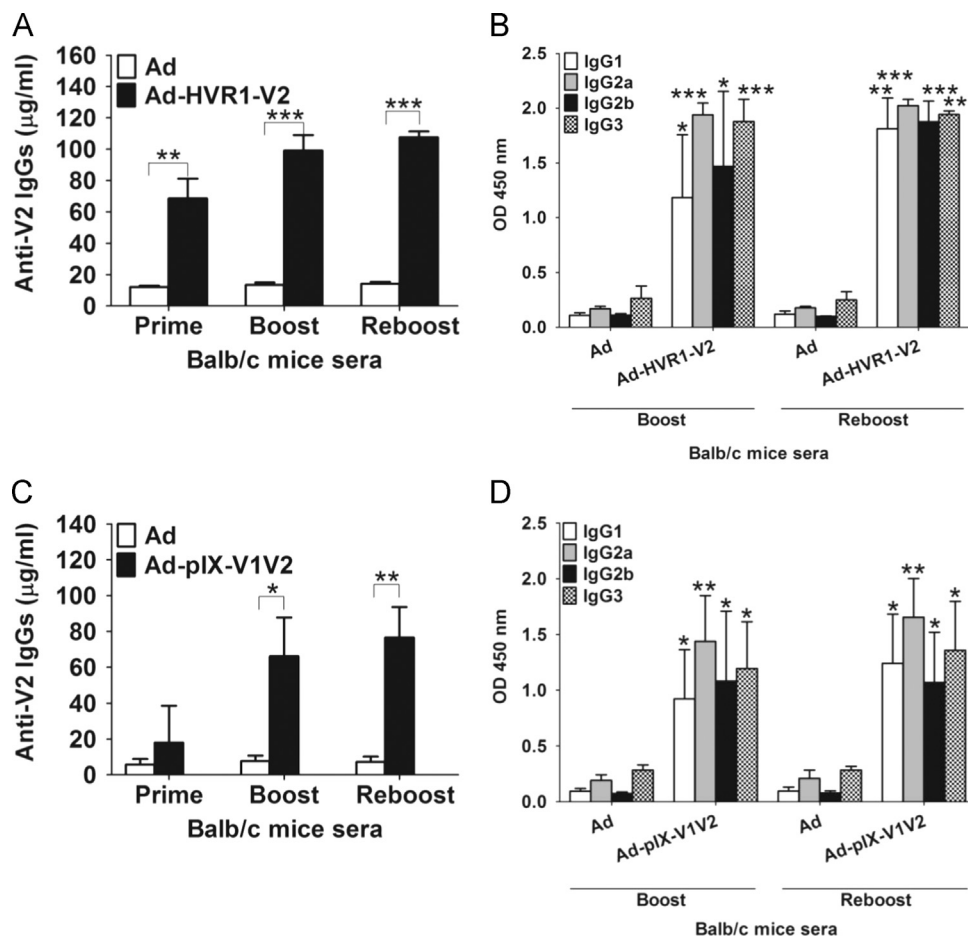


Fig. 5. V2-specific conformational IgGs elicited by Ad-HVR1-V2 or Ad-pIX-V1V2. (A) Balb/c mice sera were collected from immunizations with Ad-HVR1-V2. In the sera-based total IgG ELISA, cyclic V2 peptide was immobilized in the ELISA plates to titrate the V2-specific total IgGs (conformational) in sera collected from post prime, boost and reboost time points. (B) Corresponding to the total IgG ELISA, cyclic V2 peptide was immobilized in the ELISA plates in the sera-based IgG isotype ELISA. Goat anti-mouse isotypes, IgG1, IgG2a, IgG2b and IgG3 were then applied, followed by incubation with HRP-conjugated donkey anti-goat antibody. (C) Similarly, Balb/c mice sera were collected from immunizations with Ad-pIX-V1V2. Cyclic V2 peptide was immobilized in the ELISA plates in the sera-based total IgG ELISA to titrate the V2-specific total IgGs (conformational) in sera collected from post prime, boost and reboost time points. (D) Cyclic V2 peptide was immobilized in the ELISA plates in the sera-based IgG isotype ELISA. Goat anti-mouse isotypes, IgG1, IgG2a, IgG2b and IgG3 were then applied, followed by incubation with HRP-conjugated donkey anti-goat antibody. All data were expressed as the mean \pm standard deviation, from four independent replicates.

significant induction of antibodies specific to the V2 peptides when compared to unmodified Ad immunizations. Of these, the Ad-HVR1-20 aa-V2 vector induced comparatively low binding activity to the linear 20aa-V2 peptide (Fig. 3B), while Ad-pIX-V1V2 induced comparatively high binding activity to both the linear and cyclic V2 peptides (Figs. 4C, 5C) and Ad-HVR1-V2 induced the highest response to the both V2 peptides (Figs. 4A, 5A). The various immune response levels among these three vectors could be explained by the incorporation of different regions of V1V2, consistent with findings from the RV144 trial. The hotspot analyses in this trial found that the most immunodominant domain (15aa peptide) of clade C V2 is located in the crown/middle region of the V2 loop (Zolla-Pazner et al., 2013). In the V2 sequence of Z205F strain, the most immunodominant domain is “TEIRDKNRKEYALFY” as underlined in green, and the sub-immunodominant domain is “RDKNRKEYALFYRPD” as underlined in orange (Fig. 1A). The 20 aa-V2 clade C sequence (underlined in black in Fig. 1A) in Ad-HVR1-20aa-V2 is located on the carboxyl terminus of the V2 loop, which is poorly immunogenic. Both the V2 sequence in Ad-HVR1-V2 and the V1V2 sequence in Ad-pIX-V1V2 contain the immunodominant domain, which may have contributed to the high level of V2-specific immune responses generated by both viral vectors. The V2-specific antibodies from Ad-HVR1-V2 immunizations were generated at a higher magnitude at every time point as compared to the antibodies generated from Ad-pIX-V1V2 immunizations

(Fig. 4A and C, 5A and C). This difference could be in part due to the fact that pIX has only one third of the molecular copies of hexon on each adenoviral virion. The varying immune response levels to V2 peptides among the three viral vectors were further confirmed by an immunofluorescence assay, which showed that reboost sera from Ad-HVR1-20aa-V2 immunized mice failed to bind the HIV-1 Z205F Env protein (Fig. 7B), whereas reboost sera from Ad-pIX-V1V2 immunizations did bind to this protein at a moderate level (Fig. 7D). Moreover, boost sera from Ad-HVR1-V2 immunizations showed low binding that was enhanced by the reboost sera (Fig. 7C). The lack of binding to the Z205F Env by sera from Ad-HVR1-20aa-V2 immunized mice could be explained by (1) selection of mouse strain for immunization (2) poor immunogenicity of the 20aa-V2; (3) the fact that both key residues, asparagine at aa¹³⁴ of V1 loop and arginine at aa¹⁸⁹ of V2 loop (indicated with asterisks in Fig. 1A), are required for the generation of V2 conformational antibodies, similar to 6.4 C (Lynch et al., 2011). The 20aa-V2 only contains arginine at aa¹⁸⁹; (4) the 20aa-V2 might not be accessible on the Z205F Env trimer, which needs crystal structure analysis in the future. The detection of sera binding to the Z205F Env protein from Ad-HVR1-V2 immunizations was prior to the detection in Ad-pIX-V1V2, which is consistent with the results of sera binding to V2 peptides.

As stated elsewhere, the antibodies specific against both linear and discontinuous V2 epitopes correlated with reduced risk of

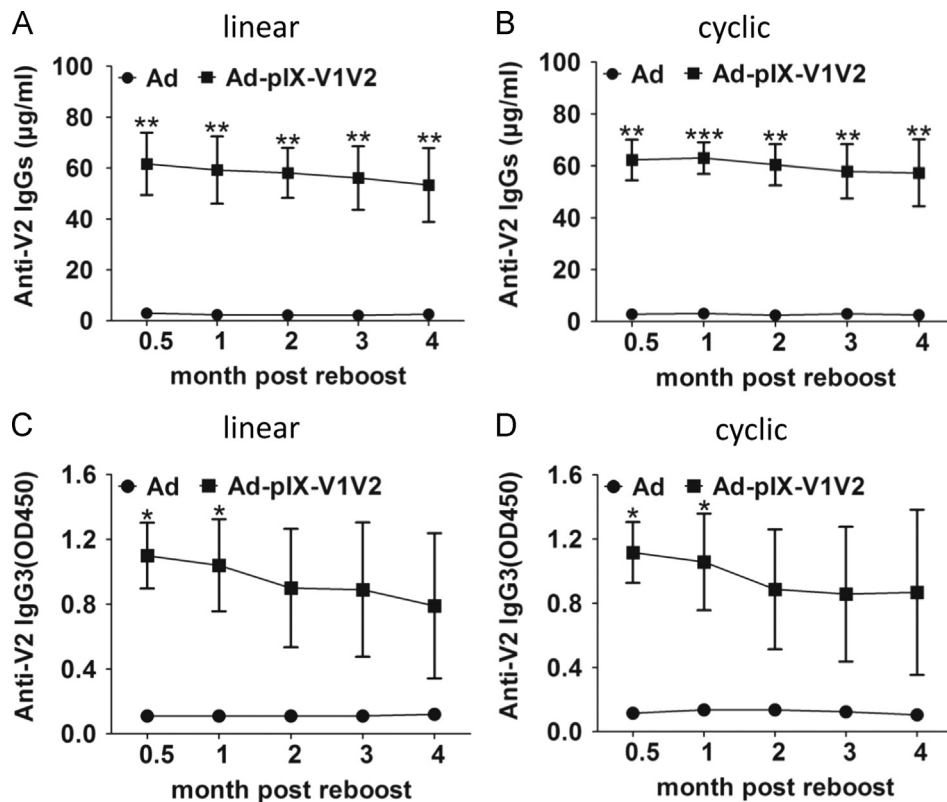


Fig. 6. Vector immunizations induced durable total V2-specific humoral immunity, but not V2-specific IgG3. In the sera-based total IgG ELISA, linear V2 peptide (A) or cyclic V2 peptide (B) was immobilized in the ELISA plates to titrate the V2-specific total IgGs in sera collected from post reboost time points (0.5, 1, 2, 3 or 4 months). In the sera-based IgG3 ELISA, linear V2 peptide (C) or cyclic V2 peptide (D) was bound to ELISA plates. Sera from post reboost time points (0.5, 1, 2, 3 or 4 months) were bound to the plates. Goat anti-mouse isotypes, IgG3, was then applied, followed by incubation with HRP-conjugated donkey anti-goat antibody. All data were expressed as the mean \pm standard deviation, from four independent replicates.

HIV-1 infection in the RV144 trial (Gottardo et al., 2013; Haynes et al., 2012). In our study, both Ad-HVR1-V2 and Ad-pIX-V1V2 elicited robust levels of anti-V2 antibodies targeting both linear V2 epitopes (Fig. 4A and C) and discontinuous V2 epitopes (Fig. 5A and C), which is informative. To our knowledge, this is the first time this result has been shown in the context of V2 in combination with the “Antigen Capsid-Incorporation” strategy on Ad vectors. Ideal vaccines should provide lasting and effective immune protection. Our data demonstrated that the total IgGs, specific to either linear (Fig. 6A) or discontinuous (Fig. 6B) V2 epitopes, were relatively stable for four months post reboost. This finding implied that Ad vectors displaying V2 loops could potentially induce durable humoral immunity specific to V2. Since the V2-specific IgG3 isotype in the RV144 trial correlated with reduced HIV-1 acquisition, but possessed a short half-life (Yates et al., 2014), we investigated the V2-specific IgG3 duration in the mouse model, using Ad-pIX-V1V2. The results demonstrated that IgG3 was significantly induced at boost, and effectively maintained until two months post reboost (Figs. 4D, 5D, 6C and D). This finding was consistent with the result in the RV144 trial. Thus, developing the mechanisms to increase IgG3 half-life might improve the HIV-1 vaccine efficacy.

Conclusion

Overall, this study is the first to demonstrate the utility of HIV-1 clade C V2 incorporation with the “Antigen Capsid-Incorporation” strategy. This study demonstrates V2 exposure, antigenicity, and durable antibody responses generated in mice using two distinct Ad5 capsid locales. As a proof-of-principle, this study

forms a foundation for more stringent development of V2 vaccine candidates in combination with rare serotype adenoviral vectors and the “Antigen Capsid-Incorporation” strategy.

Materials and methods

Construction of recombinant Ad5 vectors

To construct two versions of Ad5 vectors displaying different sizes of V2 of HIV-1 gp120 onto the hexon HVR1 locale of the vector capsid, two DNA fragments were synthesized and subcloned into HVR1 (a locale replaced from amino acids 139 to 144) of shuttle plasmid H5/pH5S (Wu et al., 2005). The two fragments are 20aa-V2 (DIVPLKPNDNSNSREYILINC) and full length of V2 (CSFNATTEIRDKNRKEYALFYRPDIVPLKPNDNSNSREYILINC), respectively. The resulting plasmids HVR1-20aa-V2/pH5S and HVR1-V2/pH5S were digested with EcoRI and PmeI. These resulting fragments containing the homologous recombination regions and the hexon genes were recombined through homologous recombination with a Swal-digested Ad5 backbone lacking the hexon gene, pAd5/ Δ H5 (Wu et al., 2002). The recombination was performed in *Escherichia coli* BJ5183 (Stratagene, CA), leading to the identification of positive vector clones, respectively. To construct Ad-pIX-V1V2, full length of V1V2 sequence (CSNYNNCNDTYS-TANCTSGGEIKNCNFNATTEIRDKNRKEYALFYRPDIVPLKPNDNSNSREYILINC) was subcloned into the pIX shuttle vector to generate pIX-shuttle-V1V2. The resulting plasmid was then digested with PmeI, the digested fragment containing the homologous recombination regions and the pIX gene was recombined through homologous recombination with an Ad5 backbone lacking the pIX

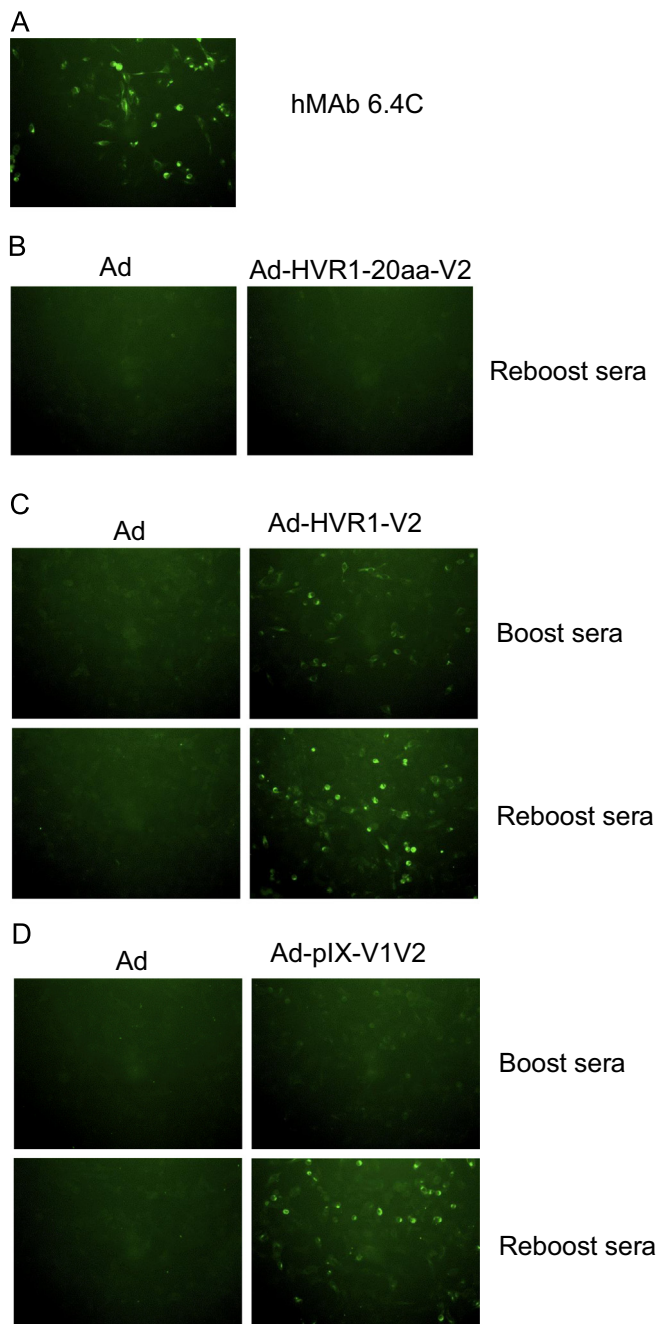


Fig. 7. V2-specific mice sera bind to HIV-1 Z205F Env. Immunofluorescence assay was employed to evaluate whether sera from V2 immunizations could bind to HIV-1 Z205F Env. (A) The positive control hMAb 6.4 C was applied to cells expressing HIV Env. (B) Reboost sera from Ad or Ad-HVR1-20aa-V2 immunized mice were applied to cells expressing HIV-1 Env. (C) Boost and reboost sera from Ad or Ad-HVR1-V2 immunized mice were applied to cells expressing HIV-1 Env. (D) Boost and reboost sera from Ad or Ad-pIX-V1V2 immunized mice were applied to cells expressing HIV-1 Env.

gene. The recombination was performed in *Escherichia coli* BJ5183 (Stratagene, CA), leading to the identification of positive vector clones.

Rescue, purification and titration of recombinant Ad5 vectors

To rescue vectors, the recombinant adenoviral genomes were digested with PacI, and transfected with PolyJet™ in vitro DNA transfection reagent (SigmaGen Laboratories, MD). Transfections were performed in Ad5-E1-expressing HEK293 cells. Multi-step

large-scale propagations of recombinant Ad5 vectors were performed after the vectors were rescued. To purify the rescued vectors, two-step cesium chloride ultracentrifugation was employed, followed by dialysis against 1x PBS containing 10% glycerol. To titrate the purified vectors, physical titers, expressed as viral particles (VPs) per ml were measured using absorbance at 260 nm. The infectious particles (IPs) per ml were determined by TCID₅₀ assay.

Native western blot

To analyze both the exposure and antigenicity of V2 on viral vectors, vectors (7×10^9 VP for Ad, Ad-HVR1-20aa-V2 and Ad-HVR1-V2, and 2.1×10^{10} VP for Ad-pIX-V1V2) were mixed with 4x native loading buffer without boiling, and resolved on native PAGE gels, followed by transfer and blocking on PVDF membrane. Blotting was performed with HIV-1 205 F strain-derived hMAb 6.4 C (1:1,000), and with goat anti-human IgG-HRP secondary antibody (1:2000; Southern biotech, Birmingham, AL). The proteins were detected by using 3'3'-diaminobenzidine tablets (Sigma-Aldrich, MO) (Gu et al., 2013).

Whole virus ELISA

The whole virus ELISAs were performed as described elsewhere (Gu et al., 2013), in order to investigate both the exposure and antigenicity of V2 on surface of capsid. Briefly, different amounts of vectors (2×10^9 VP/well for Ad-HVR1-20aa-V2 and Ad-HVR1-V2, and 6×10^9 VP/well for Ad and Ad-pIX-V1V2) were immobilized and blocked. The immobilized vectors were incubated with hMAb 6.4 C (1:1,000), followed by incubation with goat anti-human IgG-HRP secondary antibody (1:2000; Southern biotech, Birmingham, AL). ELISAs were developed with the SIGMAFAST OPD peroxidase substrate (Sigma-Aldrich, MO) and measured at OD 450 nm.

Sera-based total IgG ELISA and IgG isotype ELISA

Sera-based total IgG ELISA was performed to determine the V2-specific total IgG antibody binding responses in the mice sera. In brief, V2-derived linear or cyclic peptides were immobilized on ELISA plates at 10 μ M. Sera were sequentially diluted and incubated for 2 h, followed by a 2 h incubation with goat anti-mouse IgG-HRP secondary antibody. ELISA analyses were measured as described above.

Sera-based IgG isotype ELISA was performed to determine the V2-specific IgG isotype distributions in the humoral immune responses. Briefly, V2-derived linear or cyclic peptides were immobilized at 10 μ M. Sera were sequentially diluted, added to the plates, and incubated for 2 h. Then the plates were incubated with goat anti-mouse isotypes, IgG1, IgG2a, IgG2b and IgG3 (1:1,000; Sigma-Aldrich, MO), followed by incubation with HRP-conjugated donkey anti-goat antibody (1:5,000). Then ELISAs were measured as above.

Mice immunization

Mice immunizations with vectors (Ad, Ad-HVR1-20aa-V2 and Ad-pIX-V1V2) were performed to determine the V2-specific immunogenicity. Female Balb/c or C57BL/6 mice (6–8 weeks) in groups of eight were intramuscularly immunized with the corresponding vector (1×10^{10} VP/mouse) at each time-point, with two-week intervals among prime, boost and reboost, or between injection and bleeding. The University of Alabama at Birmingham Institutional Animal Use and Care Committee approved the use of

mice as described herein under the approved protocol number 101109272.

Immunofluorescence assay (IFA)

HEK293 cells were seeded in a 96-well plate and cultured overnight to achieve a monolayer density at 80%. Plasmid pcDNA3.1-Env (Rong et al., 2009), was designed to express the Env protein cloned from a clade C HIV-1 infected subject Z205F (Z205FPL27MAR03ENV6.3, Genbank accession number GQ485419) and was transfected into the HEK293 cells with PolyJet™ in vitro DNA transfection reagent. Transfection was carried out according to the manufacturer's manual. At 24 h post transfection, cells were rinsed once in PBS, fixed with 100 µl/well of cold fixation solution (Acetone: Ethanol [3:2]) for 5 min at room temperature, and air-dried at room temperature. Fixed cells were then rinsed in PBS, and incubated with mice sera (1:50) from prime, boost and reboost, or hMAb 6.4 C (1:500) at room temperature for 2 h. For the secondary antibody, rabbit anti-mouse IgG-FITC (1:100; Biolegend, CA) was applied corresponding to the mice sera, and goat anti-human IgG (H+L)-FITC (1:50; SouthernBiotech, AL) was applied corresponding to hMAb 6.4 C. After an incubation of 40 min at 37 °C, cells were washed in PBS, and observed for FITC under a fluorescence microscope (Nikon eclipse TE2000-5). All fluorescent pictures were taken with 20x objective lens.

Statistical analyses

The data were presented as the mean ± the standard deviation. Statistical analyses were performed with the nonpaired two-tailed Student *t*-test, assuming unequal variance. Statistical significance was defined as $p \leq 0.05$.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LLG, VK and AK performed experiments. LLG, VK, AK, AF, CD and QM contributed to the experimental design and results discussion. LLG and QM wrote the manuscript. All authors read and approved the final manuscript.

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